The Use of Epigenetic Biomarkers for Preclinical Detection of Hepatocellular Carcinoma: Potential for Noninvasive Screening of High-Risk Populations

 Commentary on Zhang et al., p. 2378

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In this issue of Clinical Cancer Research, Zhang et al. (1) examined the potential for the use of epigenetic biomarkers in a noninvasive approach to early detection of hepatocellular carcinoma in a population of high-risk patients from Taiwan. Using DNA prepared from serum samples corresponding to hepatocellular carcinoma patients, but collected at various time intervals before the diagnosis of cancer, these investigators examined methylation of promoter sequences for p16, p15, and RASSF1A. These genes are subject to methylation-dependent inactivation (silencing) in various forms of cancer, including hepatocellular carcinoma (2). Identification of methylated DNA sequences in serum that correspond to cancer-related epigenetic events in occult tumors has been suggested as a potentially valuable biomarker for preclinical detection of malignant lesions (3). In fact, several studies of hepatocellular carcinoma have shown that methylated p16, p15, and RASSF1A sequences are present in serum at the time of cancer diagnosis (4–6). Thus, aberrantly methylated promoter sequences corresponding to these genes represent potentially valuable epigenetic biomarkers for early (preclinical) diagnosis of hepatocellular carcinoma. Using a group of hepatocellular carcinoma patients from a high-risk population (and a corresponding group of control individuals from the same high-risk population), Zhang et al. (1) were able to detect hypermethylation of p16, p15, and RASSF1A in 44%, 24%, and 70% of hepatocellular carcinoma patients, respectively. Furthermore, methylated sequences corresponding to these genes could be detected in serum 1 to 9 years before the clinical diagnosis of cancer (1). The results of this investigation show the potential power for the use of sensitive and specific epigenetic biomarkers in serum as a noninvasive strategy for screening individuals at high risk for development of hepatocellular carcinoma. This testing methodology could find immediate practical clinical application for the routine screening and monitoring of individuals in regions with high hepatocellular carcinoma incidence, such as Eastern Asia and sub-Saharan Africa. In addition, given the worldwide trend toward increases in hepatocellular carcinoma prevalence in Europe, Australia, and the United States, this approach might find widespread utility in the routine surveillance of patients with defined risk factors around the world.

Hepatocellular Carcinoma: A Global Health Problem

Hepatocellular carcinoma represents the fifth most common form of neoplastic disease worldwide (accounting for >5% of all human cancers) and the third most common cause of cancer-related death (7, 8). The worldwide distribution of hepatocellular carcinoma reflects regions of high incidence and regions of low incidence. The world regions of highest incidence of hepatocellular carcinoma include Eastern Asia and sub-Saharan Africa (7, 8). The highest rates of hepatocellular carcinoma have been documented in Mongolia, with 99 cases per 100,000 population (7). In contrast, North and South America, Northern Europe, and Australia have very low incidence of hepatocellular carcinoma, with <10 cases per 100,000 population (7, 8). The worldwide variation in incidence of hepatocellular carcinoma largely reflects variations in risk factors for the resident populations, particularly exposure to hepatitis viruses (9, 10). Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) increases the risk of development of hepatocellular carcinoma. Recent worldwide estimates of hepatitis virus infection suggest that 370 million individuals are HBV positive and that 130 million people are infected with HCV (11). Other risk factors associated with the development of hepatocellular carcinoma include gender, dietary factors (including alcohol consumption and exposure to aflatoxin B1), other environmental exposures, and various genetic (metabolic) diseases (10, 12). All of these risk factors contribute to chronic liver disease, which may be the most important determinant of liver cancer risk. In world regions that have the very highest prevalence of hepatocellular carcinoma, the at-risk population is HBV positive and exposed to dietary aflatoxin B1 (13). Thus, among these people, chronic liver injury related to HBV occurs in the presence of the potent direct-acting hepatocarcinogen aflatoxin B1 (14). In the past, hepatocellular carcinoma was considered to be a major problem only in specific regions of Eastern Asia and sub-Saharan Africa. Recent trends suggest, however, that the incidence of hepatocellular carcinoma is increasing worldwide, particularly in countries and world regions that were considered to be low-prevalence areas (15). If these trends persist, hepatocellular carcinoma will emerge as a major form of
malignant cancer in the United States in the coming decades (16–19). The dramatic recent increase in hepatocellular carcinoma in the United States (and other low-prevalence regions) is attributed primarily to HCV infection, although increases in HBV infection and chronic alcoholic liver disease may also contribute (17). The continuing high incidence of hepatocellular carcinoma in Eastern Asia and sub-Saharan Africa, combined with increasing incidence in other parts of the world, suggests that this disease will continue to represent a global health problem far into the future.

**Hepatocellular Carcinoma: A Challenge for Clinical Cancer Treatment**

Treatment of hepatocellular carcinoma presents significant challenges to the clinical oncologist, as these tumors are largely refractory to chemotherapy and are often diagnosed after the initiation of local tumor spread or distant metastasis. Thus, prevention of hepatocellular carcinoma represents the best opportunity for reduction of the worldwide burden of this disease on the human population. Although efforts are under way to reduce the number of individuals at risk for development of hepatocellular carcinoma (through vaccination against HBV), a tremendous number of people are currently at elevated risk for hepatocellular carcinoma due to chronic hepatitis (related to HBV or HCV infection or alcohol consumption) and/or cirrhosis. Given the strong association between these known etiologic agents, chronic liver disease (hepatitis and cirrhosis), and progression to hepatocellular carcinoma, individuals (and groups) with known risk factors represent populations that could be monitored on a regular basis to detect early cancerous lesions. Detection and diagnosis of hepatocellular carcinoma at an early stage (when surgical intervention is possible) may significantly improve the survival of patients with this disease. Currently used methods for detection of liver tumors, however, rely largely on radiographic imaging techniques that are not practical for population-based screening. Thus, considerable effort has been expended toward identification of practical approaches for noninvasive detection of hepatocellular carcinoma. The ideal biomarker for this type of application is one that can be detected with good sensitivity in a biological sample from the patient in a noninvasive manner (e.g., blood, sputum, or urine). For hepatocellular carcinoma, blood represents the best source for detection of cancer-related biomarkers. It is well known that many types of tumors shed cells and cellular material (including DNA) into the blood. Therefore, examination of blood-derived samples (like DNA from serum) represents a cogent strategy for development of a sensitive test for occult liver neoplasms. Several strategies have been used for the detection of occult cancers using DNA from blood (or other noninvasive source), including analysis of gene mutations and detection of microsatellite instability (20). Zhang et al. (1) used detection of methylated gene sequences in DNA from serum to detect subclinical hepatocellular carcinoma. Aberrant DNA methylation is a feature of many forms of human cancer (21). Methylated gene sequences in serum represent epigenetic biomarkers that can be applied to the detection of cells with aberrant genomes, in this case hepatocellular carcinoma.

**Utility of Epigenetic Biomarkers for Detection and Diagnosis of Hepatocellular Carcinoma**

The molecular pathogenesis of hepatocellular carcinoma involves well-defined genetic and epigenetic alterations (22). Aberrant DNA methylation represents a major form of epigenetic alteration observed in these cancers. Several DNA methylation-dependent epigenetic events have been characterized in hepatocellular carcinoma, involving a significant number of genes (2), most of which are involved in cellular growth control. Among these genes are the cyclin-dependent kinase inhibitors, p15 and p16 (23), and RASSF1A, which is suggested to function as a tumor suppressor gene (24). Not only are these genes (p16, p15, and RASSF1A) frequently methylated in hepatocellular carcinoma, several studies have shown that methylated sequences corresponding to these genes are present in serum samples at the time of cancer diagnosis (4–6). This observation suggests that methylated DNA sequences that are shed from tumors into the blood represent potentially valuable epigenetic biomarkers for detection of occult liver neoplasms. Zhang et al. (1) explored this concept to determine if epigenetic biomarkers can be detected in DNA from serum of patients before the diagnosis of cancer. In fact, these investigators found methylated alleles of p16, p15, and RASSF1A in 44%, 24%, and 70% of hepatocellular carcinoma patients (Fig. 1), and these methylated alleles could be detected 1 to 9 years before the clinical diagnosis of cancer (1). In many cases, methylated alleles were detected in the first serum samples collected from patients, whereas, in other cases, methylated alleles appeared later in testing (on subsequent sampling) or an accumulation of methylated alleles was observed over time (1). In all cases where multiple samples were collected over time, genespecific methylation patterns were preserved, suggesting a strong association between the aberrant methylation event and cancer development. The sensitivity of detection of methylated sequences corresponding to p15, p16, and RASSF1A varied among these patients examined. A conservative estimate of the sensitivity for detection of occult liver neoplasms is reflected in the average time before clinical diagnosis of cancer that methylated alleles of these genes were detected in serum samples from hepatocellular carcinoma patients: p16, 4.3 years before diagnosis (range, 1-8 years); p15, 3.4 years before diagnosis (range, 2-5 years); and RASSF1A, 4.4 years before diagnosis (range, 1-9 years). In addition to exhibiting great sensitivity, detection of methylated sequences corresponding to the selected genes occurred infrequently among individuals from the control group. Methylated alleles for p16, p15, and RASSF1A were detected in 4%, 0%, and 6% of control DNA samples (1). This suggests that there is a low level of methylated sequences for these genes in serum from patients without diagnosed hepatocellular carcinoma. Because the control population carries many of the same risk factors as the patient population (including HBV and/or HCV infection), additional time will be required to see if any of these control individuals develop hepatocellular carcinoma. The results of Zhang et al. (1) establish this group of genes as good (potentially useful) epigenetic biomarkers for detection of hepatocellular carcinoma. The predictive power of
this group of genes (when applied together), however, exceeds that of the individual genes (Fig. 1). In 88% of hepatocellular carcinoma patients, a methylated allele corresponding to at least one of these genes is detected and 76% of patients have two methylated alleles (Fig. 1). Methylation of all three genes, however, is rarely found. Likewise, when the hepatocellular carcinoma patients and the control group are considered together \((n = 100)\), detection of any one methylated gene sequence produced 90% correct assignments and detection of any two methylated gene sequences produced 87% correct assignments. In contrast, detection of methylated alleles corresponding to specific individual genes showed lower predictive power \((p16, 70\% \text{ correct assignments}; p15, 62\% \text{ correct assignments}; and\)
RASSF1A, 82% correct assignments; ref. 1). These observations highlight the significant predictive power of these epigenetic biomarkers for preclinical detection of hepatocellular carcinoma.

Potential for Screening of High-Risk Populations

The natural history of hepatocellular carcinoma unfolds over many years in patients with chronic liver disease and involves numerous genetic and epigenetic events (22). The occurrence of these genetic and epigenetic events is well documented in hepatocellular neoplasms but are less well characterized in preneoplastic liver lesions. It is conceivable that methylation-dependent epigenetic events occur in chronic liver disease and/or in the preneoplastic (cirrhotic) liver, in addition to hepatocellular carcinoma (Fig. 2). The long interval for the development of hepatocellular carcinoma and the series of genetic and epigenetic events that occur over that period of time combine to present opportunities for early detection of liver neoplasms or preneoplastic lesions. Zhang et al. (1) present an intriguing study that shows the potential and promise of screening high-risk populations for preclinical detection of occult liver neoplasms using epigenetic biomarkers. The diagnostic approach suggested is attractive in that it does not require invasive techniques to collect DNA samples, it uses a PCR-based molecular assay (which is rapid, sensitive, and specific), and it is based on a set of genes that have excellent predictive power for hepatocellular carcinoma (1). In a practical sense, this molecular diagnostic approach will be more economically feasible than the currently used radiographic techniques, allowing for frequent repeat analysis of high-risk patients over time. Furthermore, the sensitivity for detection of the epigenetic biomarkers described likely exceeds that of the radiographic techniques by several orders of magnitude. Nevertheless, some questions remain and additional investigation is warranted. Detailed gene methylation studies will be required to determine if these genes (p16, p15, and RASSF1A) are methylated with high frequency in hepatocellular carcinomas related to various etiologic mechanisms. Such studies may also identify other genes that are subject to frequent methylation-dependent silencing in hepatocellular carcinoma and might represent additional valuable epigenetic biomarkers for preclinical testing. As additional epigenetic biomarkers are discovered, it will be essential to rigorously establish the sensitivity and specificity for each marker. Both positive and negative predictive value for these markers should be determined. It is essential that the epigenetic biomarkers that are applied to preclinical testing of high-risk individuals exhibit good sensitivity so that small, early neoplastic lesions can be detected. It is equally important for these markers to reflect cancer-related alterations, however, rather than epigenetic events associated with chronic liver disease that do not predict progression to hepatocellular carcinoma. Ultimately, Zhang et al. (1) provide an excellent starting point for additional prospective studies aimed at determining whether treatment of patients with subclinical disease benefit from chemopreventive or chemotherapeutic intervention before the onset of hepatocellular carcinoma.

References

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